Anti-fungal substances of *Bacillus subtilis* against *Ganoderma boninense*

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ABSTRACT

Aims: To determine the optimum culture incubation time for β -glucanase and chitinase production by *Bacillus subtilis* as well as optimum pH and temperature condition for enzymatic activity against *Ganoderma boninense*. The suitable solvent (methanol, ethyl acetate or hexane) for the extraction of bacterial metabolites from *B. subtilis* were also determined.

Methodology and results: *In vitro* antagonistic activity of antifungal metabolites derived from *B. subtilis* to inhibit the growth of *G. boninense* was evaluated based on time of culture incubation, extraction solvent of the metabolites, and enzymatic treatments conditions including pH and temperature. The results showed that β -glucanase could be optimally produced (with a specific activity 4.222 U/mg-protein) after 28 h of incubation. The optimum pH and temperature for the activity of β -glucanase were 7.5 and 45 °C respectively when 1% laminarin used as the substrate. *B. subtilis* showed optimum chitinase activity (0.0514 U/mL) after 8 h of incubation. Optimum pH and temperature of chitinase were at pH 6.0 and 40 °C, respectively using 1% colloidal chitin as the substrate. β -glucanase crude enzyme showed strongest antifungal activities against the mycelial growth of *G. boninense* better than crude enzyme of chitinase with an inhibition rate of 47.75% at 5 days of incubation. Furthermore, cultivation of *B. subtilis* over 48 h produced antifungal metabolites which could inhibit the growth of *G. boninense* the most. The best solvent to extract metabolite from *B. subtilis* was identified as ethyl acetate that rendered an inhibition value of 38.91%.

Conclusion, significance, and impact of study: *Bacillus subtilis* could be a potential biological control agent against *G. boninense*.

Keywords: *Bacillus subtilis*, *Ganoderma boninense*, antifungal, β -glucanase, chitinase

INTRODUCTION

Ganoderma boninense is a fungal pathogen in oil palm plantations that causes basal stem rot (BSR) disease which is challenging to be eradicated (Hushiaran *et al.*, 2013). Biological disease control has been used to prevent, mitigate or particularly control plant diseases (Pal and Gardener, 2006). *Bacillus* spp. have shown their ability for controlling a wide variety of fungal infections, operating as an antagonist towards fungal plant pathogens through their production of a several antifungal and antibacterial metabolites such as zwittermicin A, kanosamine and lipopeptides from iturin, surfactin and fenzycin families (Kumar *et al.*, 2009) and hydrolytic enzyme that degrade fungal structure polymers (e.g. β -glucanase, chitinase) (Chang *et al.*, 2007).

Many studies have reported the potential of *Bacillus subtilis* as a biological control agent against fungal pathogens (Arras, 1993; Arras and Dhalewin, 1994; Islam *et al.*, 2012; Mardanova *et al.*, 2017). A strain of *B. subtilis* was found to produce some volatile compounds and could inhibit the soil born plant pathogens such as *Rhizoctonia solani* and *Pythium ultimum* (Fiddaman and

Rossall, 1993). *In vitro* antagonism assay showed that *B. subtilis* PY-1 was effective against *Fusarium oxysporum* and many other agricultural fungal pathogens which has a great potential as an agent for biological control of many fungal diseases (Gong *et al.*, 2006). Meena *et al.* (2016) also reported that the production of both Cyclic lipopeptides (CLPs) iturin A, and fengycin which were produced by *B. subtilis* ABS-S14, inhibited the growth of green mold fungi (*Penicillium digitatum*), whereas surfactin did not affect straight to *P. digitatum*. In another study case, antagonism of *B. subtilis* strain AG1 was tested against vine wood fungal pathogens as well as *Phaeoacremonium aleophilum*, *Phaeomoniella chlamydospora*, *Verticillium dahliae* and *Botryosphaeria rhodiana*. The assay indicated that metabolites of *B. subtilis* strain AG1 against the growth of all pathogens were tested (Alfonzo *et al.*, 2009).

Glucan is a linear polymer of carbohydrates composed of glucose monomers with β -1,3 and β -1,6-glucan bonds, whereby these polymers are present in fungal pathogen cell walls after chitin (Budiarti *et al.*, 2004). β -glucanase is a hydrolytic enzyme which can degrade glucan compound or laminarin as its substrate to

glucose monomer or saccharide oligomer (Pitson *et al.*, 1993). β -glucanase and chitinase enzymes producing rhizobacteria could inhibit the growth of fungal pathogens that contain glucan and chitin compounds in their cell wall (Benitez *et al.*, 2004; Cota *et al.*, 2006). Chitin is reputed to be the second most abundant polymer after cellulose. Chitinase is a group of enzymes that can degrade chitin polymer with two stages. Endochitinase (EC 3.2.1.14) degrades the polymer into oligomers then the oligomer is degraded to monomers by exochitinase (β -N-acetyl hexosaminidase (EC 3.2.1.52) (Liu *et al.*, 2010).

Chitinase is used as a biocontrol against various types of fungal pathogens, which is expected to reduce the use of synthetic fungicides (de la Vega *et al.*, 2006). Several studies on the ability of *Bacillus* sp. to produce chitinase that breaks the chitins to produce N-acetylglucosamine in cultured media (Trotta *et al.*, 1973; Karunya *et al.*, 2011; Senol *et al.*, 2014; Cheba *et al.*, 2016). In fact, the enzymes productions and their reactions depend on more factors than temperature, pH and components of growth media.

MATERIALS AND METHODS

Sample collection

Ganoderma boninense and *B. subtilis* strain E-1 16S rRNA gene (JN648100) cultures used in this study were previously isolated from rhizosphere soil of oil palms in Kalimantan, Indonesia in 2012 by Ajidarma (2012). *Ganoderma boninense* culture was grown on Potato Dextrose Agar (PDA) plates and the fungal culture was used during 3 to 7 days of cultivation at 27 ± 2 °C. The bacterium *B. subtilis* strains E-1 was grown and maintained on nutrient agar (NA) medium as a stock culture at 27 ± 2 °C.

Determination of *B. subtilis* growth and β -glucanase production

The bacterium *B. subtilis* (2-4 loopful) were prepared into 50 mL media containing: K_2HPO_4 (0.1%), Ammonium sulfate (0.7%), Triptone (0.1%), NaCl (0.1%), $MgSO_4 \cdot 7H_2O$ (0.01%), yeast extract (0.05%) supplemented with 1% laminarin and incubated on shaker incubator at 37 °C and 170 rpm agitation until the optical density number was adjusted to 1×10^8 spores/mL. Furthermore, 1% inoculum of active culture was cultivated into 250 mL Erlenmeyer flask containing 100 mL of enzyme production media (mentioned above) supplemented with 1% laminarin at pH 7.0. The culture was collected every 4 h until 48 h to measure optical density at 580 nm. The culture was then centrifuged at $3500 \times g$ for 30 min at 4 °C to obtain the crude extract of the β -glucanase enzyme (Wijaya, 2015). After centrifugation, the supernatant of β -glucanase was then measured for its activity by the Somogyi-Nelson method (Natsir, 2014) and protein content analysis conducted (Bradford, 1976).

Assay of β -glucanase activity and protein concentration

β -glucanase activity was determined by measuring the reducing sugar hydrolysis of laminarin as a substrate (Wijaya, 2015). The crude extract (250 μ L) was incubated at 37 °C for 30 min with 1% laminarin (250 μ L) in 50 mM pH 5.2 sodium acetate buffer. The result of the enzyme hydrolysis was reacted with the 500 μ L reagent of Nelson A and Nelson B reagent, it followed by heating in a 100 °C water-bath for 20 min. The reaction was then allowed to cool as well as the room temperature (at 25 °C). Addition, 500 μ L of arsenomolybdate reagent was added, homogenized and waited for 5 min. The absorbance was measured at a wavelength of $\lambda=540$ nm. The amount of reducing sugar released was calculated using glucose as standard.

Characteristic of β -glucanase activity by pH and temperature

β -glucanase production was characterized by a different range of pH and temperature. The effect of pH on β -glucanase activity was determined by measuring the different range of pH values (pH 5.0 to 8.0) using 1% laminarin as a substrate under standard assay conditions. The buffers used were 50 mM sodium acetic acid buffer (pH 5.0 and 5.5), and 50 mM sodium phosphate buffer (pH 6.0 to 8.0). The optimum temperature was determined by incubating the reaction mixtures at a different temperature from 30 to 55 °C with 5 °C intervals, using the previous observation that pH optimum of activity. The stability of β -glucanase activity was correlated by incubating the enzyme activity within optimum pH and temperature for 30 min (Wijaya, 2015).

Determination of *B. subtilis* growth and chitinase production

The active bacterium *B. subtilis* (1% inoculum) was cultivated into 250 mL Erlenmeyer flask containing 100 mL of nutrient broth enzyme production medium with 1% colloidal chitin at pH 7.0. Each culture was incubated at 37 °C at 170 rpm in shaker incubator and collected every 4 h until 48 h. Addition, their optical density was measured at 600 nm. The culture was then centrifuged at 3,500 rpm for 20 min at 4 °C to obtain the crude extract (supernatant) of extracellular chitinase enzyme (Purkan *et al.*, 2016). The crude chitinase was then measured for its activity.

Assay of chitinase activity and protein concentration

Chitinase activity was carried out according to Purkan *et al.* (2016). The crude extract of an extracellular enzyme (400 μ L) was added with 1% colloidal chitin substrate (1600 μ L) which was dissolved in 50 mM pH 7.0. The homogenized samples were then incubated at 37 °C for 30 min. Chitinase activity was determined by measuring absorbance at 660 nm and using colloidal chitin as a

standard. Measurement of enzyme activity was finalized by using the turbidimetric method. The calculation was: The colloidal chitin concentration = colloidal chitin concentration before hydrolysis – colloidal chitin concentration after hydrolysis. Protein concentration was determined by Bradford method (1976) using bovine serum albumin as standard.

Characteristic of chitinase activity by pH and temperature

The effect of pH on chitinase activity was determined by measuring the activity at 30 °C and the pH range of (pH 5.0 to 8.0) using 1% colloidal chitin as a substrate under standard assay conditions. The following buffers used were: 50mM sodium citrate buffer (pH 5.0 and 6.0), and 50 mM sodium phosphate buffer (pH 6.0 to 8.0). The optimum temperature was determined by incubating the enzymatic reaction mixtures within the temperature range of 20 to 60 °C with 10 °C intervals, using the previous observation that pH optimum of activity. The stability of chitinase activity was investigated by incubating the enzyme activity within optimum pH and temperature for 30 min (Purkan *et al.*, 2016).

In vitro antagonistic activity assay by the crude enzyme of β -glucanase and chitinase production

In vitro assay was performed using agar well diffusion method to detect which antifungal enzyme (β -glucanase and chitinase) were active against *G. boninense*. These assessments were comprised of two parts: In the first part, crude extract of β -glucanase (100 μ L) or crude extract of chitinase (100 μ L) was transferred into the wells, which far away from center of a fresh PDA plates (2.5 cm), and an agar plug (6 mm diameter) of *G. boninense* was then placed centrally on a PDA plate, which the wells containing antifungal enzymes. One well containing sterile distilled water had used as negative control and one well containing salicylic acid (0.4 mg/mL) was used as positive control. Subsequently, antagonistic activities of β -glucanase or chitinase enzymes were assessed qualitatively. In the second part, these entire tests for antagonistic activity were incubated at room temperature and it was possible to observe in all growth directions as well as daily visual observation until day 5 of incubation. To identify a potential role of both enzymes to inhibit the growth of *G. boninense* was assessed for their antagonistic effect on *G. boninense* within all parameters that were investigated by measuring the mycelium of *G. boninense* colony in the direction towards the antagonist colony (R2). The data were later transformed into percentage inhibition of radial growth (PIRG) in relation to the radial growth of *G. boninense* in the control plate (R1) which would be estimated by the following equation:

$$\text{PIRG (\%)} = \frac{R_1 - R_2}{R_1} \times 100$$

Where, R1 is the length of radial growth of fungus (2.5 cm) in control sets and R2 is the radial growth of the fungus-treated with antagonistic activity (Bivi *et al.*, 2010). The experiment was conducted around 5 times.

Culture media and bacterial metabolite extraction from *Bacillus subtilis*

The active inoculums of *B. subtilis* (10%) were incubated into fresh 50 mL (w/v) of nutrient broth (NB) mediums with a pH 7.0 in 100 mL Erlenmeyer flasks and incubated on a temperature shaker at 37 °C, 150 rpm (ZHWY-200B/200D). Fifty milliliter culture broth from 3 replicates were collected for 28 h, 48 h, 72 h and 96 h of incubation. The cell culture was harvested by centrifugation at 4500 rpm for 40 min at 4 °C by Ramyabharathi and Raguchander (2014). Different type of solvents (methanol, ethyl acetate, and hexane) was used for extracting antifungal metabolites from the bacterial cell-free supernatant. A total of 5 mL of cell-free supernatants were combined with 5 mL of each solvent (1:1 ratio) to a final volume of 10 mL in 25 mL falcon tubes. Subsequently, these samples were incubated at room temperature for overnight with shaking at 150 rpm speed. After the overnight incubation, the extraction of all supernatants with a mixture of each solvent was centrifuged at 4500 $\times$ g for 40 min at 4 °C and transferred into 15 mL falcon. After centrifugation, all the solvent extracts that contained metabolite compounds were directly assayed for their antifungal metabolite activity, an *in vitro* assay.

In vitro antifungal activities of bacterial metabolite extract from *B. subtilis* against *G. boninense*

The antifungal metabolites activity of *B. subtilis* was obtained by using the extraction method of maceration. According to Lenny (2006) maceration is done by soaking the samples in an organic solvent at a certain time at room temperature. The process is commonly used to extract metabolites compounds. The basic principle of maceration is soaking the sample in solvents which will lyse the cells because of the differences of pressure inside and outside of the cells. Thus, metabolites produced by *B. subtilis* could dissolve in solvents.

The effectiveness of all the solvent extracts was assayed for their antifungal activity by paper disc diffusion method (Alemu and Alemu, 2013). A mycelial plug of 6 mm diameter from 3 days old of *G. boninense* culture was cut and transferred to the center of PDA plates. The sterile filter paper discs were immersed in solvent extracts for 20 min and additionally placed onto PDA plates which containing the fungal pathogen. The filter paper discs were placed in 2.5 cm away from the agar plug of the fungal pathogen. One filter paper disc without solvent extracts were used as a control. All the plates were incubated at room temperature for 4 days of incubation. The inhibition of antifungal activity was investigated as a zone of mycelial growth of *G. boninense* on PDA plates, the inhibition zones were visibly observed as well as daily

visual observation until day 4 of incubation. The percentage of inhibition of radial growth was estimated following the equation below:

Delta Inhibition Diameter (Δ) = SD – ED

Net Inhibition Diameter (NID) = NCD – ED + Δ

$$\text{Net Inhibition (NI)\%} = \frac{\text{NCD} - \text{NID}}{\text{NCD}} \times 100$$

$$\text{PIRG (\%)} = 100 - \text{NI}$$

Where SD is Solvent Diameter; ED is extraction diameter, and NCD (Negative Control Diameter) is a length of radial growth of fungus (2.5 cm) in control sets.

Statistical analysis

The data were statistically analyzed for significance by ANOVA using Minitab version 6.0 systems for Windows and a value of $p \leq 0.05$ was considered statistically significant.

RESULTS

Bacillus subtilis growth and enzyme production

Bacillus subtilis growth and β -glucanase production

The determination of optimum condition β -glucanase activity was done by testing some variables: measurement of *B. subtilis* growth, measurement of protein content and testing β -glucanase activity against incubation time fermentation (enzyme production). From the experiment, it was found that the OD₆₀₀ of *B. subtilis* culture started increasing exponentially from 0.0475 to 0.7655 at adaptation phase on the hour-0 until 8 h of incubation (Figure 1). Subsequently, the growth increased up to 28 h at an OD₆₀₀ of 1.484 and slowly decreased at 48 h of incubation. The growth rate of *B. subtilis* was suspected to decrease, due to a progressive decrease in substrate availability per cell over time caused by microbial consumption.

In addition to the β -glucanase enzyme, *B. subtilis* produced β -glucanase enzyme at the beginning of cell growth and the highest enzyme activity increased when cells entered the stationary phase (Figure 1). Dewi *et al.*, (2017) reported that β -glucanase produced by *B. subtilis* SAHA 32.6 was secreted in the log phase when the media contains glucose and exhibited high activity when bacterial cells entered the stationary phase, which contains glucan as the carbon source. β -glucanase activity increased when cells stopped growing after lack of glucose. β -glucanase activity increased only when cells stopped growing during glucose or amino acid starvation (Stulke *et al.*, 1993). In this experiment, the β -glucanase enzyme of *B. subtilis* was secreted after 4 h of incubation (2.110 U/mg) and increased to 3.174 U/mg after 20 h of incubation. The specific β -glucanase enzyme activity increased to 4.222 U/mg after 28 h of incubation and declined steadily (Figure 1). Therefore, the addition of

glucose (yeast extract and tryptone) and glucan (laminarin) as substrate caused β -glucanase activity to be the highest at stationary phase. The result in Figure 1 showed that the highest β -glucanase activity by *B. subtilis* was achieved at 28 h of incubation. So, the time was used for the cultivation process to harvest the enzyme and to find out the optimum β -glucanase activity at different pH and temperature.

Figure 1: Production of β -glucanase and protein concentration by *B. subtilis* on β -glucanase medium containing 1% laminarin at 37 °C and pH 7.0.

B. subtilis growth and chitinase production

The growth and production of chitinase by *B. subtilis* was investigated in nutrient broth (NB) growth media which contained 1% colloidal chitin as substrate (pH 7.0) at 37 °C on the shaker incubator at 170 rpm. The crude enzyme was analyzed for chitinase activity every 4 h until 48 h incubation. The growth rate of *B. subtilis* strains E-1 increased from 0 to 8 h of incubation. At that time the growth was relatively stable up to 16 h of incubation and decreased after 16 h of incubation (Figure 2). This result was supported by Hogg (2005) who stated that the increase in the number of cells during bacterial growth indicated that the bacteria utilized the nutrient source in the medium as a component which was required for its growth, while the decrease in the number of bacterial cells in the growth curve occurred after the active phase of bacterial growth (called stationary phase) because the bacteria will produce secondary metabolites that are toxic.

The chitinase enzyme activity after 4 h of incubation was 0.0312 U/mL and relatively increased to 0.0514 U/mL after 8 h of incubation. The enzyme activity descended after 8 h of incubation. The result in Figure 2 showed that the chitinase enzyme activity was high before the cell growth entered the stationary phase. Consequently, this study presented that *B. subtilis* could produce chitinase enzyme at the early stage of fermentation in NB growth media which contained 1% colloidal chitin as the carbon source. The chitinase activity decreased before the cell

entry to stationary phase. Jholapara *et al.* (2013) reported that colloidal chitin served as the best carbon source to produce chitinase, meanwhile another carbon source such as glucose served to inhibit chitinase biosynthesis despite supporting prosperous cell growth of the *B. subtilis* in media. Thus, NB media was used for bacterial cell growth, but it was not fulfilling all requirements necessary for antibiotic production. However, the highest chitinase activity was found after 8 h of incubation.

Figure 2: Production of chitinase and protein content produced by *B. subtilis* on NB medium containing 1% colloidal chitin at 37 °C and pH 7.0.

Characteristic of β -glucanase and chitinase activity by pH and temperature

*Effect of pH value on the crude enzyme of β -glucanase and chitinase activity of *B. subtilis**

The characterization of β -glucanase and chitinase crude extracts by pH and temperature were described in Figures 3 and 4. Effect of pH on the activity of β -glucanase enzyme has shown that the specific enzyme activity increased with the increasing pH up to pH 7.5 (Figure 3). The specific of β -glucanase activity at pH 5.5 was 4.25 U/mg which rose sharply to 6.36 U/mg at pH 7.0 and then continued increasing until pH 7.5 with an activity of 7.04 U/mg which was considered the optimum pH of the β -glucanase of crude enzyme. Furthermore, with increasing pH > 7.5, specific β -glucanase activity was decreased to 4.75 U/mg at pH 8.0.

Based on the result of this experiment, it could be pointed out that pH 7.5 was the optimum pH of β -glucanase activity. The previous study reported by Wijaya (2015) demonstrated that the β -1,3 glucanase activity from *B. licheniformis* BKA53 has an optimum pH 7.5 and optimum temperature at 45 °C. Beauvais *et al.* (2001) also reported that the hydrolysis enzyme which degrades β -1,3 glucanase can generally work in the neutral pH conditions by breaking down a glucan to a polysaccharide made of several glucose sub-units. This shows that, high pH would influence reaction products, and inhibits the β -

glucanase reaction. Changing the pH value will not only affect the shape of β -glucanase, it can also cause changes to the charge properties of the substrate (laminarin). Consequently, either enzyme substrate could not bind to the active site or it could not undergo catalysis (Nielsen *et al.*, 2001 and Ali *et al.*, 2014). In this experiment, a pH 8.0 may affect dramatically both the enzyme conformation and substrate properties.

Figure 3: Effect of different pH value on the specific activity of β -glucanase by *B. subtilis*. The activity was measured at 37 °C.

Effect of the pH on chitinase activity was shown in Figure 4. The result showed that the pH increased the specific activity of the crude enzyme up to a maximum of 0.011 U/mg-protein at pH 6.0, but the enzyme activity decreased in pH higher than pH 6.0. Purkan (2016) reported that the chitinase activity by fungi *Aspergillus niger* has optimum pH at pH 6.0 and the optimum temperature of 40 °C. The enzyme activity was determined by the presence of donor protons and donor acceptors on the degree of ionization, obtained at optimum pH reported (Wilson and Walker, 2005). Moreover, the pH also affects the solubility of the substrate and Farabee (2001) reported that extreme pH caused changes in the structure of the enzyme and then decrease the effectiveness and efficiency of enzyme activity.

*Effect of temperature on β -glucanase and chitinase activity of the crude enzyme of *B. subtilis**

Temperature is one of the factors that affect the enzyme activity. Changing temperature can lead to protein or enzyme folding so that the enzyme active site is in the right position to catalyze substrate.

The experiment result showed that the specific activity of β -glucanase increased at 35 °C with a specific activity of 0.726 U/mg-protein, rising to 0.763 U/mg at 40 °C and further to 1.680 U/mg at 45 °C. The specific activity of the enzyme decreased with increasing temperature to 50 °C (0.717 U/mg) and declined steadily to 0.351 U/mg at 55 °C (Figure 5). Increasing the incubation temperature of an

enzyme could cause the increase of enzyme activity. This is due to the increased speed of thermal motion of enzyme molecules, which increased the numbers of molecules that have enough energy to reach the transition state (Bollage and Edelstein, 1991). Based on the data, it can be concluded that the maximum specific activity of β -glucanase at 45 °C was observed when hydrolyzing laminarin substrate.

Figure 4: Effect of different pH value on the specific activity of chitinase by *B. subtilis*. The activity was measured at 37 °C.

Figure 5: Effect of different temperature value on the β -glucanase activity of the crude enzyme by *B. subtilis*. The activity was measured at pH 7.0.

The chitinase activity of the crude enzyme at various temperatures increased dramatically with evaluation of temperature up to a maximum activity of 0.045 U/mg protein at 40 °C, but the activity of the enzyme was reduced when the temperature increased (Figure 6). According to Lestari *et al.* (2017), the optimum temperature of chitinase activity from *B. subtilis* B 298 was achieved at 40 °C. The similar optimum temperature was obtained for the chitinase activity of *Bacillus* sp. R2 (Cheba *et al.*, 2016) and *Pseudomonas aeruginosa* K-187 (Wang and Change, 1997). The optimum temperature of chitinase activity from *B. subtilis* is not sufficiently high; this is because chitinase is classified as the mesophilic enzyme (Fardiaz, 1989). So, the result of chitinase activity can be assumed as not resistant to heat.

Figure 6: Effect of different temperature value on chitinase activity of *B. subtilis*. The enzyme activity was determined at pH 6.0.

In vitro* antifungal activity on *G. boninense* by β -glucanase and chitinase enzyme from *B. subtilis

The effectiveness of antagonist activity against the mycelial growth of *G. boninense* by β -glucanase and chitinase enzyme from *B. subtilis* was investigated according to the best incubation time and physical characteristics of β -glucanase and chitinase products from the previous experiment. *B. subtilis* produced highest β -glucanase activity at 28 h incubation, and the optimum pH and temperature of β -glucanase were 7.5 and 45 °C respectively. Meanwhile, *B. subtilis* produced highest chitinase activity at 8 h incubation. With an optimum pH of 6.0 and temperature of 40 °C.

Inhibition of the growth of fungal pathogen of *G. boninense* was detected *in vitro* assay by using the crude enzyme (100 μ L) of β -glucanase and (100 μ L) chitinase using an agar well-diffusion method. The experiment results showed that the β -glucanase of crude enzyme significantly inhibited the mycelial growth of *G. boninense* on agar plates after 3 days of incubation and increased inhibition until 5 days of incubation (Figure 7).

In contrast, the ability of crude chitinase inhibited the growth of *G. boninense* until 4 days of incubation by 10.74% when compared with control and decreased after 5 days of incubation (Figure 7). Furthermore, chitinase was not significantly different ($P > 0.05$) in inhibiting the growth of *G. boninense* compared with control. It was allegedly because of the low activity of chitinase enzyme, and the cell wall of *G. boninense* also contained glucan and other compounds, which could block chitinase enzyme in hydrolyzing or degrading the cell wall of *G. boninense*.

The effectiveness of the β -1,3-glucanase enzyme is related to the ability of an enzyme that only specifically degrade the glucan molecules of the fungal cell wall. Therefore the ability of β -1,3-glucanase enzyme to degrade the cell wall components is weakened. As a result, the growth inhibition effect to *G. boninense* has not maximized. Wrobel *et al.* (2014) reported that besides glucans and chitins, fungal pathogen cell walls also contain other components such as proteins and lipids

which cover the cell wall of fungi that can block the degradation of the main components of fungal cell wall. For this reason, the effectiveness of β -glucanase and chitinase to inhibit the growth of *G. boninense* was decreased after 5 days of incubation on PDA agar medium.

Yurnaliza (2001) also stated that chitinase enzyme activity of Actinomycetes RKT-5 showed no inhibition growth of *F. oxysporum* because the components of the fungi cell wall were not entirely chitin, consequently chitinase was unable to hydrolyze other cell wall components such as glucan or proteins. There were three factors, which can influence the effectiveness of lytic enzyme of Actinomycetes RKT-5 in inhibiting the growth of *F. oxysporum*. These factors were low enzyme concentration; the fungal cell walls contain proteins and lipids that inhibited chitinase activity and loss or appearance of other compounds that play a role during the test. Thus, the specific activity of chitinase was low since the cell wall of *G. boninense* also contained glucan, proteins, and lipids. Consequently, the inhibition rate of chitinase on *G. boninense* growth *in vitro* assay was also low.

The finding of this study concluded that the crude enzyme of β -glucanase could inhibit the growth of *G. boninense* better than the crude enzyme of chitinase, 47.75% compared to 1.12% inhibition, respectively, which was compared with control after 5 days of incubation (Figures 7-9). It showed that β -glucanase was more

effective in inhibiting *G. boninense* compared to chitinase as the specific activity of β -glucanase was higher than chitinase in this study. According to Fesel and Zuccaro (2016) chitins are located at the apex and in the inner parts of the lateral wall, so it is more susceptible to chitinase at the tip than in the subapical part. Whereas, glucans are located on the outer layer where they are degraded by β -glucanase (Kim and Yun, 2006).

This experiment was comparable to Vidhyasekaran (1997) results which reported that β -1,3 glucanase inhibited the growth of *F. solani* fsp. *sipi* at 250 μ g/mL, while chitinase did not inhibit the fungi. The results suggested an absolute requirement for that concentration of enzyme activities to cause inhibition of the fungi, which may occur in infected pea pod tissues. Accordingly, the concentration of specific activity of β -glucanase was higher than chitinase, 1.680 U/mg to 0.045 U/mg, respectively.

Result of extraction and the effectiveness of antifungal metabolite from *B. subtilis*

Antifungal metabolites had been successfully extracted from the bacterial cell culture at 28 h, 48 h, 72 h and 96 h incubation in NB at 37 °C, pH 7.0. Madigan *et al.* (2012) suggested that bacteria started producing metabolites in the last stationary phase of bacteria growth. Metabolite extraction was done by using different solvents namely methanol, ethyl acetate, and hexane.

Figure 7: Inhibition of the mycelial growth of *G. boninense* by β -glucanase and chitinase of *B. subtilis* strains E-1 on PDA medium (after 2-5 days of incubation). Sterile distilled water as a Negative control and Salicylic acid (0.4 mg/mL) as positive control.

Figure 9: Antagonistic activity of β -glucanase and chitinase derived from *B. subtilis* against mycelium of *G. boninense*. Growth inhibition of fungal mycelia was examined in dual culture assay on agar plates at 5 days of incubation: (N) negative control; (P) positive control; (G) β -glucanase enzyme; (C) chitinase enzyme.

Figure 10: Antifungal metabolites extracted from 48 h, 72 h and 96 h cultures of *B. subtilis* strains E-1 against *G. boninense*. Growth inhibition of fungal mycelia was examined in dual culture assay on agar plates at 4 days of incubation. (C) Control; (M) methanol with metabolites extracted; (E) ethyl acetate with metabolites extracted; (H) hexane with metabolites extracted.

Figure 11: Comparison of antifungal metabolites produced by difference time of *B. subtilis* cultures to inhibit the mycelial growth of *G. boninense* on PDA plates at 27 ± 2 °C. Growth inhibition of fungal mycelia was examined in dual culture assay on agar plates at 4 days of incubation.

The results showed that metabolite extracts of *B. subtilis* produced clear inhibition zone (Figure 10) with the highest inhibition of *G. boninense* growth was obtained at 48 h cultures and metabolites extracted by ethyl acetate solvent which gave 38.91% inhibition after 4 days incubation (Figure 11). Ridwan (2005) reported that the solvent acetone and ethyl acetate were common solvents used in the extraction process which play a role as semi-polar solvents. Ramyabharathi and Raguchander (2014) isolated an efficient strain of *B. subtilis* EPC016 from the cotton plant that produced both surfactin and Iturin antifungal compounds, would play a major role in *F. wilt* suppression.

In another study by Darwiati (2009) stated that the compounds with the same polarity will be easily dissolved in a solvent which has similar polarity. The previous study by Chen and Juang (2008) also reported that ethyl acetate is the best extraction of surfactin which had a recovery of 99% and resulted in purify of 60%, whereas methanol and hexane are less suitable for amounts of surfactin are solved when compared with ethyl acetate solvent. Hence, in these study metabolites compounds contained in *B. subtilis* were trended to semi-polar components. Thus, the ethyl acetate solvent showed highest attached metabolites of *B. subtilis* to inhibit the growth of *G. boninense* as an antifungal metabolite. However, antifungal metabolite extracted with each solvent was shown the ability to inhibit the growth of *G. boninense* when compared with its control (Figures 10 and 11).

The findings of the present study demonstrated that the maximum antifungal metabolite activity of *B. subtilis* was obtained from 48 h cultures. Although the good inhibition activity was also found at 48 and 96 h cultures. The result of 48 h cultures inhibited the mycelial growth of *G. boninense* to about 38.91%, when compared to 32.30% and 20.04% after 96 h and 72 h cultures, respectively (Figure 11). Whereas, crude antifungal metabolites extracted at 28 h of incubation, failed to inhibit the growth of *G. boninense* (data not shown).

Based on the data in Figure 11, the solvent ethyl acetate had the best ability to attach metabolite produced by *B. subtilis* when compared with methanol and hexane solvents. Thus, antifungal metabolite showed the highest activity to inhibit the mycelial growth of fungi *G. boninense* at 48 h cultures, especially metabolites that were extracted by ethyl acetate as a semi-polar solvent, followed by methanol and hexane. Three kinds of solvents showed high inhibition activity at 48 cultures and the inhibition activity was reduced at 72 h and increased again at 96 h cultures. This was the correlation that the growth of *B. subtilis* into the nutrient broth mediums influenced changing the pH of the mediums as they produced metabolites while they were growing (Figure 11). As the result showed that the inhibition activity was relatively high at 48 h and 96 h cultures and at 72 h culture was low inhibition activity, so *B. subtilis* released metabolites to neutralize the pH to its growth conditions and then started growing vigorously which made the mediums altered. Gesheva *et al.* (2005) found that the pH

changes due to the release of metabolites in nutrient broth (NB) mediums and cause the metabolite productions active against bacterial isolates. Thus, the pH could have a direct effect on the metabolite productions which produced by *B. subtilis* at difference time.

The effectiveness of antifungal metabolite extracted from *B. subtilis* against *G. boninense* was confirmed by the inhibition of the mycelial growth in different solvent extracts compared to the control on PDA plates. The results showed that the mycelial growth of fungi on the side of the control paper disc grew thicker than on the side of the paper disc containing dissolved metabolites extracted by different solvent (Figure 10). So, metabolites produced by *B. subtilis* played a role as antifungal because it inhibited the mycelia growth of *G. boninense* on PDA plates. This was supported by Leelasuphakul *et al.* (2008) statement that antimicrobial compounds and antifungals produced by microorganisms might play a role in inhibiting the growth of fungi.

CONCLUSION

The maximum inhibition rate (47.75%) of *G. boninense* by the β -glucanase activity of *B. subtilis* was achieved after 28 h of culture incubation and enzymatic treatment condition of pH 7.5 and temperature 45 °C. The maximum inhibition rate (10.74%) of *G. boninense* by chitinase activity of *B. subtilis* was achieved after 8 h of culture incubation and enzymatic treatment condition at pH 6.0 and 40 °C. Incubation of *B. subtilis* over 48 h produced antifungal metabolite which can inhibit the growth of *G. boninense* the most and ethyl acetate was the best solvent to extract the antifungal metabolites with the inhibition rate of 38.91%. It can be concluded that β -glucanase, chitinase, and metabolites produced from *B. subtilis* which have antifungal activity against *G. boninense* are expected to be used in the medical field against fungal infections and in agriculture sector as biological agents.

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